Dose-dependent acceleration in the delayed effects of neonatal oral exposure to low-dose $17\alpha$-ethynylestradiol on reproductive functions in female Sprague-Dawley rats

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ABSTRACT — Xenoestrogen exposure during the critical period of sexual differentiation of the brain causes delayed effects on female reproduction. We investigated the internal dose of orally administered ethynylestradiol (EE) during the critical period and its delayed effects by administering 0 (vehicle control), 0.4, or 2 $\mu$g/kg EE to female Sprague-Dawley rats for 5 days from postnatal day (PND) 1. Determination of serum EE level 24 hr after the initial dosing and 6 and 24 hr after the final dosing of 2 $\mu$g/kg indicated that the administered EE entered the circulation and cleared after every administration. Although the treatment did not affect physical development, including growth, eyelid opening, and vaginal opening, the estrous cycle was arrested from postnatal week (PNW) 12 even with 0.4 $\mu$g/kg EE, with an inverse correlation between doses and arresting ages. Although ovarian morphology at PNW 22-23 indicated that the treatment caused long-term anovulation and cystic follicle formation, the number of primordial follicles at PNW 22-23 was similar among the groups. Because this number was lower than that at PND 10 in all groups, primordial follicles may have been consumed under long-term anovulation. The treatment also caused other abnormalities, including mammary gland hyperplasia, increase in pituitary and liver weights, and decrease in the uterine weight. Because the highest circulating EE level in the 2 $\mu$g/kg-treated neonates is considered to be comparable to the physiological range of estradiol-17$\beta$, we concluded that a slight increase in the circulating estrogens during the neonatal period exerts irreversible delayed effects.

Key words: $17\alpha$-ethynylestradiol, Internal dose level, Critical period, Sexual differentiation, Estrous cycle

INTRODUCTION

Critical periods for morphogenesis or functional differentiation in humans and animals are highly sensitive not only to their endogenous key molecules but also to exogenous analogues for such key molecules (McLachlan et al., 2012). Among these key molecules, sex steroids are determinants of sexual phenotypes after organogenesis in vertebrates (Gore et al., 2014; Chung and Anthony, 2013), and it has been well established that exposure to xenoestrogens, such as alkylphenols, synthetic estrogens, and phytoestrogens (Jefferson et al., 2012) affects female reproductive function in rodents during the critical period of sexual differentiation (Frye et al., 2012). In a previous study, we have found that a single s.c. injection of $17\alpha$-ethynylestradiol (EE) at a dose level up to 2 $\mu$g/kg on postnatal day (PND) 1 arrests the estrous cycle at younger ages and that treatment with a lower dose of EE lasts for a longer period until the arrest of estrous cycle (Shirota et al., 2012). In that study, 0.08 $\mu$g/kg of EE at PND 1
arrested the estrous cycle in some animals at 32 weeks of age, slightly younger than vehicle-treated animals. These findings suggest that the delayed effects of weak estrogens could be escaped from toxicological studies conducted under various safety test guidelines because the guidelines do not require monitoring the estrous cycle of the treated animals until middle age. The National Institute of Environment in Japan has confirmed estrogenic activity for more than 80 compounds among 273 candidate compounds. While the estrogenic activity of these compounds, except for synthetic estrogens, is generally weak, most of these compounds are orally exposed to humans and animals through food, as food contaminants, or as food ingredients, or medicine (Safe, 2000). However, only limited studies have been conducted on the effects of oral exposure (Ohta et al., 2012; Cimafranca et al., 2010; Prins et al., 2011). Therefore, in the present study, we investigated the delayed effects of oral exposure to a low-dose estrogenic compound on reproductive functions in female Sprague-Dawley rats.

EE is a synthetic estradiol that is commonly used in formulating contraceptives. Since EE is able to exert estrogenic effects as endogenous estrogens do, it has been used as a positive control compound in evaluating the estrogenic activity of various xenobiotics. Compared with estradiol-17β, the binding affinity of EE with recombinant full-length human estrogen receptor (ER) α is estimated to be 56% (Freyberge et al., 2010). Agonistic activity of EE mediated by human ER α was estimated to be approximately 35 times higher than that by human ER β by the assay using cell-lines co-expressing a reporter and either receptor (Barkhem et al., 1998). The relative in vitro estrogenic activity of EE to estradiol-17β is varied among studies. While the reported lowest value was 50% (Nakamuro et al., 2002), most of in vitro studies estimated the value ranged 90-250% (Soto et al., 1995; Coldham et al., 1997; Fang et al., 2000; Nishihara et al., 2000; Sanseverino et al., 2009).

However, the binding affinity of EE with rat α-fetoprotein, a major transport protein found in the serum of fetal and neonatal rats (Gitlin and Boesman, 1967), is approximately 100 times lower than that with estradiol-17β (Hong et al., 2012). Therefore, orally administered EE is expected to pass through the blood-brain barrier of neonates as xenoestrogens do if EE reaches the circulation. Therefore, in the present study, we determined the circulating level of EE after dosing to provide information on the relationship between the internal dose of EE and delayed effects for further evaluation of the delayed effects of xenoestrogens.

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MATERIALS AND METHODS

Test compound
EE (CAS #57-53-6, Sigma-Aldrich Japan, Tokyo, Japan) was dissolved in ethanol (Wako Pure Chemical Industries, Osaka, Japan) at a concentration of 100 mg/mL, following which the solution was diluted with corn oil (Wako Pure Chemical Industries) to formulate EE at a constant volume of 10 mL/kg.

Doses of EE
Doses of EE in the present study were set at 0.4 and 2 μg/kg/day because we had already confirmed that s.c. injection of 0.4 and 2 μg/kg of EE significantly alters the estrous cycle. Therefore, we adopted identical dose levels to the previous study at a daily dose or an accumulated dose for 5 days.

Experimental design
Procedures for animal experiments described below have been approved by the Committee of Animal Experiments at Azabu University. Female neonates were obtained through spontaneous delivery from pregnant Sprague-Dawley rats [Crl:CD(SD)IGS], which were purchased from Charles River Japan (Kanagawa, Japan) or were obtained by mating with adult male Crl:CD(SD)IGS rats purchased from the same breeder. The animals were maintained under a lighting condition (lights on: 08:00-20:00) in an animal husbandry facility, which was controlled for the standards of temperature and humidity at 21 ± 1°C and 50-60%, respectively. The animals were housed in a plastic cage with bedding materials (Sunflake; Oriental Kobo, Tokyo, Japan) with pellet chow (CE-2; Clea Japan, Tokyo, Japan) and tap water (Kanagawa Prefectural Government, Kanagawa, Japan) ad libitum. The pregnant animals were daily monitored for delivery from gestational day 20, and the day when neonates were confirmed by 16:00 was designated as PND 0. The neonates were collected and assigned to each treatment group at PND 1. This study consisted of main and satellite experiments. The main experiment was performed to examine delayed effects of orally administered EE from PND 1 to 5, the period of steroid sensitivity for female fertility (Barraclough, 1961; Gorski, 1968), using 45 female neonates from 7 dams. The satellite experiment was performed to determine the circulating EE levels after the initial and the final oral administration of EE to 32 female neonates from 7 dams. All groups of the neonates were identified by a tattoo, and those in the main study were placed under the same dams to equalize maternal effects on them. The litter size was adjusted to 8 female neonates.
in the both experiments if possible. When the numbers of female neonates in dams were less than 8, male offspring were placed under the dams to adjust the size. There were two exceptions in the litter size; 10 female neonates were placed under one dam in the main experiment, and 13 female neonates were placed under one dam in the satellite experiment until blood collection on PND 2.

In the main experiment, EE was orally administered for 5 days from PND 1 at a dose level of 0 (corn oil 10 mL/kg), 0.4, or 2 μg/kg using an intubation tube prepared according to Watanabe et al. (2009). In the satellite experiment, EE was orally administered at PND 1 or for 5 days from PND 1 using the same method as that used in the main study at a dose level of 2 μg/kg. The body weight of the neonates was individually measured daily during the administration period in the both experiments and weekly measured from PND 7 and on the day of necropsy in the main experiment. Their general condition was observed as well.

In the satellite experiment, blood was collected from the hearts or abdominal vessels at 24 hr after the first dosing at PND 1 or at 6 or 24 hr after the final dosing at PND 5, respectively, from 8-14 animals. The collected blood was centrifuged at 2,500 rpm for 25 min at 4°C, following which serum was collected and kept at -50°C until determination of the EE concentration.

In the main experiment, at PND 10, 4-5 animals from each group under 2 dams were killed by decapitation. Then, their ovaries were collected. The collected ovaries were fixed in Bouin’s solution to determine the number of primordial follicles. Animals under the other dams were determined for the age at eyelid opening, and were weaned at PND 21. From PND 28, the animals were observed for vaginal opening. For animals found to have a vaginal opening, the body weight was measured as well. From postnatal week (PNW) 8, the animals in each group were periodically monitored for the estrous cycle by observing the vaginal cytology for 2 weeks at 2-week intervals until PNW 21.

Necropsy was performed at PNW 22-23 on the day of estrus if at all possible. For necropsy, animals were killed by bleeding from the abdominal aorta under anesthesia with sodium pentobarbital (Somnopentyl; Schering-Plough Animal Health, Osaka, Japan). Following necropsy, the pituitary gland, mammary glands, and major thoracic and abdominal organs or tissues were dissected. Among these, mammary glands were fixed in a phosphate-buffered 10% formalin solution, and the ovaries were fixed in Bouin’s solution. Oviducts were collected to determine ovulation using the method of Burdick and Whitney (Burdick and Whitney, 1941). These tissues were dehydrated and embedded in paraffin for further examination.

### Determination of serum EE concentrations

The serum EE concentration was determined by ultra-fast liquid chromatography (Prominance; Shimadzu Scientific Instruments, Kyoto, Japan) and triple quadrupole mass spectrometry (API 5000; AB Sciex Ltd., Framingham, MA, USA) at Sumika Chemical Analysis Service, Ltd. (Osaka, Japan) according to the methods described by Borgers et al. (2009). Prior to analysis, serum samples collected from 3-5 animals after the initial treatment or from 2-3 animals after the final treatment were pooled to 1 sample (300 μL), and 10 μL of internal standard, 50 ng/mL of ethynylestradiol-\(d_4\) solution, and 150 μL of 0.1 N hydrochloride were added to each sample. These samples were mixed with 4 mL of tert-butyl ether, following which the organic phase of the mixture was transferred into a glass tube. The tubes were evaporated to dryness and were then mixed with 150 μL of dansylchloride (1 mg/mL in acetone) and 150 μL of carbonate pH standard solution (pH 10.01) to be derivatives. They were incubated for 30 min at 60°C, following which the derivatives were extracted with 3 mL of hexane. The organic phase was evaporated to dryness, and the residue was dissolved in 200 μL of acetonitrile and water mixture (1:1 v/v). The samples were applied to the system after ultrafiltration (Centricut Ultramini; Kurabo, Osaka, Japan). Chromatography and mass spectrometry conditions are shown in Table 1. In the present analysis, the lowest and highest quantification limits were 3 pg/mL and 30 ng/mL, respectively.

### Classification of estrous cycle

Estrous cycles were categorized into 3 types: 4-5-day estrous cycle, persistent estrus, and irregular cycle. That is, animals in which the estrus day revolved at 4- or 5-day intervals were classified into the 4-5-day estrous cycle, those showing no consecutive diestrus/metestrus day were classified into persistent estrus, and those not classified into either of these 2 patterns were classified into the irregular cycle. In addition, the cumulative estrus or proestrus days and the cumulative metestrus or diestrus days were calculated for each period.

### Determination of the number of primordial follicles and morphological analysis in the ovary

The paraffin-embedded ovarian tissues were serially sectioned at 5 μm thickness and were stained with hematoxylin and eosin. The number of primordial follicles was determined for ovaries from 4-5 neonates at PND 10 and...
from 3 animals at PNW 22-23 in each group according to a previously reported method (Shirota et al., 2003). Ovarian tissues showing the presence of cystic follicles and corpus luteum were observed on the serial sections in all of the animals.

**Immunohistochemistry of mammary gland**
To define the localization of mammary acini and ducts in mammary tissues from nulliparous animals, α-smooth muscle actin (SMA)-expressing cells (i.e., myoepithelial cells surrounding the acini and ducts) were detected by immunohistochemistry with anti-SMA antibody (clone 1A4; Dako A/S, Glostrup, Denmark) as described previously (Yasuno et al., 2013). The mammary tissue specimens were also stained with hematoxylin and eosin.

**Statistical analysis**
Statistical analysis was performed using JMP Statistical Analysis Software (SAS Institute, Cary, NC, USA). Initially, all data were analyzed using a one-way analysis of variance (ANOVA). Differences between the control group and any group receiving EE were analyzed by Dunnett’s test. A P value of less than 0.05 was considered statistically significant.

**RESULTS**

**Serum concentration of EE after oral administration**
The serum concentration of EE in the neonates after oral administration of 2 μg/kg of EE is summarized in Table 2. Each time point consists of 3-4 pooled serum samples from 3 neonates at PNW 22-23 in each group according to a previously reported method (Shirota et al., 2003). Ovarian tissues showing the presence of cystic follicles and corpus luteum were observed on the serial sections in all of the animals.

**Table 1.** Chromatography and mass spectrometry conditions in the determination of serum 17α-ethynylestradiol (EE) concentration.

<table>
<thead>
<tr>
<th>Ultra-fast liquid chromatography condition</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Analytical column</td>
<td>Synergi 4 μm Polar-RP 80A, 2.0 nm I.D. x 50 mm L., Phenomenex</td>
</tr>
<tr>
<td>Column temperature</td>
<td>40°C</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>A: 0.1% formic acid</td>
</tr>
<tr>
<td></td>
<td>B: 0.1% formic acid containing acetonitrile</td>
</tr>
<tr>
<td>Gradient condition</td>
<td>Time (min)</td>
</tr>
<tr>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>60</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.3 mL/min</td>
</tr>
</tbody>
</table>

**Triple quadrupole mass spectrometry condition**

| Collision gas                             | Set at 6 arbitrary unit, nitrogen |
| Scan mode                                 | Multiple reaction monitoring mode |
| Monitored ions                             | Dansyl-ethynylestradiol |
|                                           | Precursor ion: m/z 530; Production ion: m/z 171 |
| I.S. (dansyl-ethynylestradiol-\(d_4\))   | Precursor ion: m/z 534; Production ion: m/z 171 |

I.S., internal standard

**Table 2.** Serum 17α-ethynylestradiol (EE) concentration of female neonates after oral treatment with 2 μg/kg/day of EE for 5 days from postnatal day (PND) 1.

<table>
<thead>
<tr>
<th>Time after treatment</th>
<th>Number of samples</th>
<th>EE (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>At 24 hr after the initial treatment</td>
<td>4</td>
<td>12.8 ± 4.5a</td>
</tr>
<tr>
<td>At 6 hr after the final treatment</td>
<td>3</td>
<td>47.3 ± 5.2b</td>
</tr>
<tr>
<td>At 24 hr after the final treatment</td>
<td>3</td>
<td>6.7 ± 5.2a</td>
</tr>
</tbody>
</table>

Values represent mean ± S.E.M. Each sample consists of serum from 3-5 neonates at 24 hr after the initial treatment and from 2 or 3 neonates at 6 and 24 hr after the final treatment. Different characters shown in the serum EE concentration represent significantly different at P < 0.05 by the Tukey-Kramer honest significant difference (HSD) test.
samples collected from 2-3 neonates. The highest serum level among all the time points was observed at 6 hr after the final administration. The value was significantly different from those at 24 hr after the initial and the final administration; however, no significant difference was observed between those at 24 hr after the initial and the final administration.

### General condition, physical growth, and timing of vaginal opening

There was no abnormal general condition related to the EE treatment during the experimental period. No significant difference was observed in the body weight (Table 3), whereas body weight gain during PNW 12-13 in the 2 μg/kg-treated group was significantly greater than that in the control group (data not shown).

Table 4 summarizes the timing of eyelid opening and
vaginal opening of the treated animals. Eyelid opening, one of the landmarks of physical development, was confirmed at identical ages among the groups. The age at vaginal opening was slightly advanced in the 2 μg/kg-treated group; however, no significant difference was observed among the groups.

Effects on estrous cycle

Distributions of animals showing respective patterns of the estrous cycle and the ratio of cumulative number of estrus or proestrus days and that of metestrus or diestrus days are illustrated in Figs. 1 and 2, respectively. The number of estrous cycles revolved and the cumulative number of estrus or proestrus days during each observation period are summarized in Table 5. In the control group, the distribution and the cumulative number of estrus or proestrus days and that of metestrus or diestrus days were not significantly different between those at PNW 8-9 and the rest of the periods, and only 1 animal showed persistent estrus from PNW 20. In the 0.4 μg/kg-treated group, no significant differences were observed in comparison with those parameters in the control group until PNW 9. From PNW 12, however, animals in this group began to show persistent estrus (Fig. 2), and the number of estrous cycles revolved during PNW 12-13 or older ages was significantly smaller than that in the control group (Table 5). By PNW 21, none of the animals showed a normal estrous cycle. In the 2 μg/kg-treated group, none of the animals showed a normal estrous cycle at the beginning of the monitoring, and the number of estrous cycles revolved was significantly reduced at PNW 8-9.

Ovulation and reproductive organ weights at terminal necropsy

Ovulation and weights of the ovaries and uterus are summarized in Table 6. In the control group, 3 animals failed to ovulate on the day of vaginal estrus. Among these, 1 animal showed a normal estrous cycle and the other 2 showed persistent estrus or an irregular cycle by the day of necropsy. In contrast, none of the animals in the EE-treated groups showed ovulation at necropsy, and the uterine weight in the 2 μg/kg-treated group was significantly lower than that in the control group that failed to ovulate. Ovarian weights in the EE-treated groups were lower than those in the control group; however, no significant difference was observed in comparison with that in control animals that failed to ovulate.

Number of primordial follicles

As shown in Fig. 3, the numbers of primordial follicles counted at PNW 22-23 were significantly lesser than those counted at PND 10 in respective groups; however, no significant difference was observed among the groups at any age.

Ovarian histology

The summary of histological evaluation of ovaries and representative histology are shown in Table 7 and Fig. 4, respectively.

Among the ovaries of the control group (Fig. 4A), those from 2 animals that failed to ovulate lacked corpus luteum, and those from 5 animals developed cystic follicles. In the EE-treated groups (Fig. 4B), all the ovaries lacked corpus luteum, except that from 1 animal in the 0.4 μg/kg-treated group, and all developed cystic follicles.

Mammary glands

Spotted or macular accumulations of milky solution were observed beneath the skin in 2 out of 9 and 6 out of 10 animals in the 0.4 μg/kg- and 2 μg/kg-treated groups, respectively, whereas none of the animals in the control group showed such changes. Immunohistochemistry with anti-SMA antibody clearly showed myoepithelial cells around the lactiferous ducts and secretory acini of the mammary gland. In the control group, lactiferous

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**Table 4.** Ages and body weights at eyelid opening and vaginal opening of female rats orally administered 17α-ethynylestradiol (EE) for 5 days from postnatal day (PND) 1.

<table>
<thead>
<tr>
<th>Dose of EE (μg/kg/day)</th>
<th>0</th>
<th>0.4</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals examined</td>
<td>10</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Ages at eyelid opening (day)</td>
<td>13.5 ± 0.2</td>
<td>13.8 ± 0.2</td>
<td>13.7 ± 0.2</td>
</tr>
<tr>
<td>BW at eyelid opening (g)</td>
<td>34.8 ± 0.8</td>
<td>34.2 ± 0.9</td>
<td>33.4 ± 0.8</td>
</tr>
<tr>
<td>Ages at vaginal opening (day)</td>
<td>33.0 ± 0.8</td>
<td>33.1 ± 0.8</td>
<td>31.9 ± 0.8</td>
</tr>
<tr>
<td>BW at vaginal opening (g)</td>
<td>125 ± 3</td>
<td>121 ± 3</td>
<td>115 ± 3</td>
</tr>
</tbody>
</table>

Values represent mean ± S.E.M.
ducts without development of acini were scattered in the fat tissue (Fig. 5A). On the other hand, increased numbers of lactiferous ducts with developed acini were distinct in the EE-treated mammary gland (Figs. 5A and B), indicating mammary lobular hyperplasia which seems to actively secrete milk (Fig. 5C).

Table 5. Effects of neonatal 17α-ethynylestradiol (EE) exposure on the number of estrous cycles revolved and the number of estrus/proestrus days for each observation period in the female rats orally administered EE for 5 days from postnatal day (PND) 1.

<table>
<thead>
<tr>
<th>Dose of EE (μg/kg/day)</th>
<th>Number of animals examined</th>
<th>0</th>
<th>0.4</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Postnatal week (PNW)</td>
<td>The number of estrous cycle revolved</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8-9</td>
<td>12-13</td>
<td>16-17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.4 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.9 ± 0.2</td>
<td>1.6 ± 0.2**</td>
<td>0.8 ± 0.2**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1 ± 0.2**</td>
<td>0.3 ± 0.2**</td>
<td>0.2 ± 0.2**</td>
</tr>
<tr>
<td></td>
<td>The number of estrus/proestrus days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8-9</td>
<td>12-13</td>
<td>16-17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.3 ± 0.5</td>
<td>6.1 ± 0.5</td>
<td>6.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.0 ± 0.5</td>
<td>6.9 ± 0.5</td>
<td>10.9 ± 0.7**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.9 ± 0.5**</td>
<td>13.2 ± 0.5**</td>
<td>12.8 ± 0.7**</td>
</tr>
</tbody>
</table>

Values represent mean ± S.E.M. * and **, significantly different from control at \( P < 0.05 \) and 0.01, respectively.

Table 8. Pituitary weight was significantly increased in the groups administered 0.4 μg/kg or more, and liver weight was significantly increased in the group administered 2 μg/kg. Adrenal weight was also increased in the 2 μg/kg-treated group; however, no statistical difference
DISCUSSION

This study clearly demonstrated that neonatal oral exposure to EE arrests the estrous cycle even at a dose level of 0.4 μg/kg/day. The treatment increased the circulating EE levels 6 hr after the final dosing at that of estradiol-17β on the day of proestrus (Smith et al., 1975; Asai et al., 2002; Nozawa et al., 2014; Usuda et al., 2014) even at 2 μg/kg/day of EE. Because the relative in vitro estrogenic activity of EE was found to be 90-250% of estradiol-17β (Soto et al., 1995; Coldham et al., 1997; Fang et al., 2000; Nishihara et al., 2000; Sanseverino et al., 2009), estrogenic activity of the circulating EE after the administration of 0.4 μg/kg/day seemed to be equivalent to or lower than basal estradiol-17β levels in the cyclic rats. Thus, very slight estrogenic stimulus during the neonatal period exerts irreversible effects on the animals.

In the present study, we showed that repeated administration of EE at a dose level of 0.4 μg/kg/day arrests the estrous cycle from PNW 12 to 13. We have previously confirmed that a single s.c. injection of EE at PND 1 arrested the estrous cycle from PNW 20 even at a dose level of 2 μg/kg. Similar results were also reported in the study with EE doses ranging from 0.02 to 200 μg/kg at PND 0, and more than 20 μg/kg of EE affected the estrous cycle from PNW 12 (Takahashi et al., 2013). Because we confirmed that orally administered EE was cleared within 24 hr, repeated estrogenic stimulus during the neonatal period may exert greater effects on revolution of the estrous cycle. Gene expression of ERα, by which EE exerts its effects, increases in the hypothalamus during the perinatal period in female mice (Mogi et al., 2015) and female rats (Walker et al., 2012). Therefore, daily estrogenic stimulus may have a greater effect on the hypothalamus than a single administration.

The ovarian histology of EE-treated animals lacked corpus luteum and formed cystic follicles; these findings are frequently observed in the ovaries of middle-
Table 7. Incidence of animals showing absence of corpus luteum and formation of cystic follicles in ovaries and white spots in mammary glands at terminal necropsy from female rats orally administered 17α-ethynylestradiol (EE) for 5 days from postnatal day (PND) 1.

<table>
<thead>
<tr>
<th>Dose of EE (µg/kg/day)</th>
<th>0</th>
<th>0.4</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals examined</td>
<td>10</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Absence of corpus luteum</td>
<td>2</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Presence of cystic follicles</td>
<td>5</td>
<td>8</td>
<td>10</td>
</tr>
</tbody>
</table>

Fig. 4. Representative histology of ovaries collected at postnatal week (PNW) 22-23 from animals orally treated with corn oil (A) or 17α-ethynylestradiol (EE; B) for 5 days from postnatal day (PND) 1. In the ovary from the EE-treated animals, large cystic follicles share ovarian tissue, and corpus luteum was not confirmed in the tissue. Asterisks indicate corpus luteum formed at recent ovulations. Hematoxylin and eosin staining.

Fig. 5. Representative histology of mammary glands at postnatal week (PNW) 22-23 from animals orally treated with corn oil (A) or 2 µg/kg of 17α-ethynylestradiol (B and C) for 5 days from postnatal day (PND) 1. (A, B) Immunohistochemistry of α-smooth muscle actin; (C) hematoxylin and eosin staining.

Aged rats (Bukovsky et al., 2000). Some ovaries from animals in the control group developed cystic follicles and lacked corpus luteum. In the EE-treated group, however, the instances of animals showing these findings were increased in a dose-dependent manner. Because none of the animals in the EE-treated groups failed to ovulate on the day of estrus, the treatment may cause long-term anovulation, probably because of incomplete or abolished gonadotropin surges, as reported in middle-aged animals (Downsa and Wise, 2009) and in animals treated with EE s.c. at PND 0 (Usuda et al., 2014; Nozawa et al., 2014; Ichimura et al., 2015).
Despite long-term anovulation, the number of primordial follicles, a stockpile of oocytes, was decreased by aging in the EE-treated ovaries, as observed in the control ovaries. It has been reported that s.c. injection of diethylstilbestrol (DES) or bisphenol A (BPA) to female Wistar rats at 48-hr intervals from PND 1 to PND 7 does not alter the total number of oocytes but decreases the ratio of primordial follicles at PND 8. Furthermore, DES treatment increases multiple organ failure (MOF) in rats (Rodríguez et al., 2010), as found in mice treated with various ER agonists (Iguchi et al., 1990; Chen et al., 2009; Cimafranca et al., 2010). Under the present experimental condition, however, MOF was not increased in the EE-treated group, and their primordial follicle stockpile may supply to the growing phase.

In addition to effects on the reproductive organs, neonatal rats receiving EE treatment developed spotted or macular accumulations of milky solution beneath the skin. Histopathological observation revealed mammary lobular hyperplasia, which was found in the animals administered EE s.c. during the neonatal period (Shirota et al., 2012; Takahashi et al., 2013). Similar macroscopic findings have also been reported in a study in which DES was orally administered to neonatal rats (Ohta et al., 2012). However, all these previous reports confirmed the findings at elder ages than those in the present study. Repeated oral exposure to EE may advance the development of mammary alveolar hyperplasia in nulliparous rats. Because neonatal exposure to estrogenic compounds can directly cause morphological or molecular alterations in the mammary gland (Umekita et al., 2011; Betancourt et al., 2010; Ayyanan et al., 2011; Moral et al., 2011), exposure to EE during the neonatal period may exert direct effects on undifferentiating mammary tissue or may exert indirect effects via endocrinological alterations.

In conclusion, the present study clearly indicated that a slight increase in circulating estrogens during the neonatal period exerts irreversible delayed effects on females and that repeated oral exposure (the most probable route of exposure of humans and wildlife to xenoestrogens) exerts such effects.

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Conflict of interest----- The authors declare that there is no conflict of interest.

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